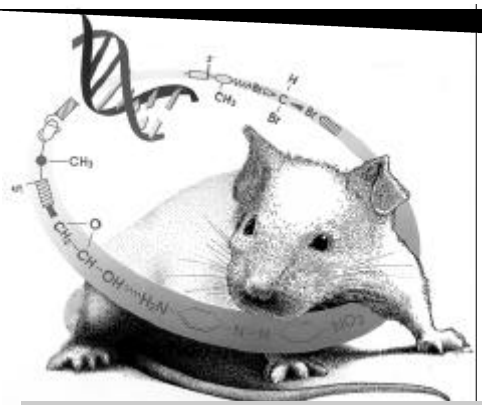


The Tg.AC Workgroup Newsletter

The Tg.AC Workgroup Newsletter is published by The Department of Toxicology and Safety Assessment, Boehringer Ingelheim Pharmaceuticals, Inc. as a means of communication for the HESI's Alternative to Carcinogenicity Testing Committee.

Letter and article submissions are welcome. Persons interested in contributing to the newsletter should contact:

Sylvia M. Furst, Ph.D.
Boehringer Ingelheim Pharmaceuticals, Inc.
Research and Development Center
900 Ridgebury Rd.
P.O. Box 368
Ridgefield, CT 06877-0368

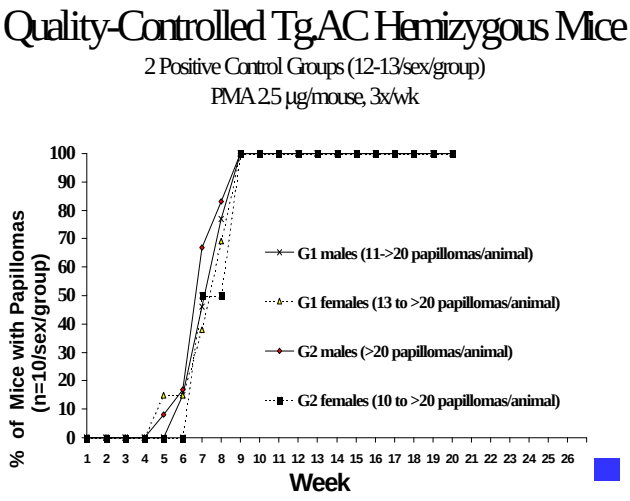
Tel: 203-791-6112
Fax: 203-798-5797
sfurst@bi-pharm.com

On the Inside:

October 28, 1998 Meeting: Update on Tg.AC Testing and Research

On October 28, 1998, a meeting was held at the FDA Woodmont facility. This meeting was called as a follow up to the March 18, 1998 meeting to discuss results of current testing and research on the Tg.AC Responder/Non-Responder genotype. A total of 16 representatives from the FDA, BIPI, Taconic, Therion , Dupont and Haskell Labs were present. The objective of the meeting was to 1) Evaluate the quality control program instituted by Taconic in order to correct the Tg.AC breeding colony and ensure production of Responder mice 2) Review research performed in order to further understand the Tg.AC Non Responder genotype mechanistically and determine further objectives for this research. A presentation was given by S. Furst (BIPI) on the results of a study performed on Tg.AC quality controlled hemizygous mice. The purpose of the study was to corroborate the response of the corrected responder Taconic hemizygous Tg.AC animals. These animals were the first produced from the reconstructed colony by the test matings of Responder breeders to FVB/NTac mice. All animals had been genotyped as responders using the zetaglobin Southern Blot technique developed by F. Sistare (FDA). A total of 50 animals were treated with PMA three times a week dermally at 2.5 ug/mouse. The study was in Drug Week 14. A 100 % response of animals was obtained by the 9th week of the study. Of the 50 animals total, 46 had greater than 20 papillomas (Fig. 1). Sam Phelan from Taconic presented the Quality Control program now in place for production of Tg.AC hemizygous mice with the Responder genotype. Generating empirical data using test mating procedures and through theoretical analysis, it was predicted that the spontaneous rate of the non responding genotype was on the order of 1-5%. Criteria were set and samples of animals at various stages of the breeding protocol would be tested for genotype as well and phenotype to ensure Responder animals. Dr. F. Sistare (FDA) presented a current summary of research on the mechanism for the alteration of the transgene resulting in the responsive/non responsive genotypes. Additional work is required to specifically define the portion of the transgene which is deleted in the Non Responder. The Southern Blot technique using the zetaglobin probe has been optimized to resolve Non Responder mice which show only small deletions in the 2 kb band. Dr. R. Cannon (NIEHS) presented work indicating that three classes of deletions exist for Tg.AC Non Responders. Retrospective analysis based on the genotype of the 25th foundation breeder pair indicated that the mutant frequency for the Responder allele to mutate to the Non Responder allele was approximately 1.5%.

Fig. 1



Contact: Dr. R. Stoll (203) 798-5684
or Dr. S. Furst (203) 791-6112

Probability curves were generated to predict the appearance of non Responder alleles in a homozygous Responder colony over multiple generations with non intervention. In conclusion, it was agreed that the quality control program now in place at Taconic with the addition of PMA phenotyping of the Pre-production colony animals by BIPI, would eliminate perpetuation of the Non Responder gene in breeders. However, animals being shipped to Tg.AC users would be subject to the 1-5% spontaneous mutation rate. Research on the mechanism for the responsive alteration of the transgene and mutation frequency would continue. Based on the data presented at this meeting, it was agreed that the hemizygous Tg.AC animals now produced from the quality controlled program are acceptable for regulatory studies.

Studies Currently Being Conducted by the Schering-Plough Research Institute at Covance Laboratories

by Margaretann Halleck, Ph.D., Schering-Plough Research Institute

As part of the HESI program, Schering-Plough is evaluating the response of Tg.AC hemizygous mice to oral (dietary) and dermal administration of phenacetin, a Group 2A carcinogen. These studies were initiated in August, 1998. We anticipate issuing final reports for both studies by the 4th quarter of 1999.

Doses for the 26-week oncogenicity studies were based on toxicokinetic and histopathologic findings in dose range-finding studies conducted in FVB/N mice. Dose-limiting histopathological findings were observed in the urinary bladder and kidneys of FVB/N mice following administration of phenacetin by either dose route.

For the 26-week dietary study, phenacetin is admixed in the feed of Tg.AC mice at concentrations of 12, 60, or 300 ppm and in the feed of FVB/N mice at a concentration of 300 ppm. One group of Tg.AC mice receives dimethylvinyl chloride by oral gavage (100 mg/kg/day, 5 days/week) as a positive control.

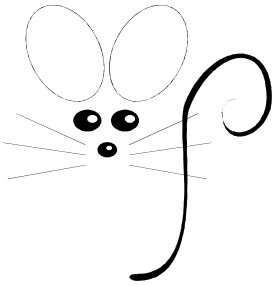
In the 26-week dermal study, the doses of phenacetin are 0.08, 0.40 and 2.0 mg/day. The vehicle is 80% acetone/20% dimethylsulfoxide and the dose volume is 200 ml. An additional group of Tg.AC mice receives tetradecanoyl phorbol-13-acetate (2.5 mg/application) three times per week as a positive control. FVB/N (wild-type) mice, dosed with 2.0 mg of phenacetin per day, are also included in the study design.

Contact: Dr. M. Halleck at (973) 579-4324
margaretann.halleck@spcorp.com

Comparative Xenobiotic Metabolism between Tg.AC and p53-Deficient Mice and their Respective Wild Types

by JM Sanders, LT Burka, JE Fossett and HB Matthews, NIEHS

The use of transgenic animals, such as Ha-ras activated (Tg.AC) and p53-deficient mice, offers great promise as a model for a more rapid and possibly more sensitive method for assessing chemical carcinogenicity. Since metabolism through reactive intermediates is a critical factor in the carcinogenic potential of many compounds, it is of interest to compare xenobiotic metabolism between transgenic animals and their corresponding wild types. Consequently, the present work has examined the comparative metabolism of three xenobiotics through a series of metabolic pathways by Tg.AC and p53-deficient mice and their respective parent strains, FVB and C57BL/6. The metabolism of each of the three substrates, benzene, ethoxyquin and methacrylonitrile has been well characterized. Together, use of these substrates offers the opportunity to examine arene oxide formation, aromatic ring opening, hydroxylation, epoxidation, O-deethylation, and a number of conjugation reactions. Most of the metabolites formed from these chemicals are excreted in urine. In the present study, excreta was collected for up to 72 hr from mice receiving single oral doses of one of the three 14 C-labeled compounds. Comparisons of elimination rates and routes and profiles of metabolites excreted in urine were performed between relevant treatment groups. Results indicted that metabolism of each of the three substrates was not altered between Tg.AC and p53-deficient mice and their respective parent strains, FVB and C57BL/6 mice.



Availability of Tissues for Special Studies

There have been inquiries about the availability of normal and tumor tissues from studies conducted as part of the ILS/ATC project for investigators who wish to conduct specific studies. For example, an academic researcher who wishes to analyze the tissues for specific changes related to tumor induction. This is an issue that must be decided ultimately by the sponsoring laboratory since their resources have been used to conduct the study. It has been proposed that sufficient tumor or target tissues be taken for histopathological evaluation and that where additional tissue is available, that those portions be quick frozen and stored at low (<-70C) temperature. If such unused fixed or frozen tissue is not needed by the sponsor it is possible that such resources could be made available for special studies by non sponsor organizations. At this time we are soliciting your opinions about making study tissues available to other investigators. Please communicate your opinions or suggestions to Dr. Denise Robinson.

Contact: Dr. D. Robinson at (202) 659-3306
drobinson@ilsi.org

Boehringer Ingelheim Starting Sulfisoxazole Tg.AC Dermal and Gavage Study

By Hank Holden, BIPI

Sulfisoxazole (CAS No. 127-69-5) is a pharmaceutical with an extensive history of clinical use as a broad spectrum bacteriostatic agent. It was selected by ILSI for testing in several of the potential alternative models for carcinogenicity detection. Previously, sulfisoxazole has been shown to be non-carcinogenic in rat and mouse two-year bioassays conducted by NCI (Technical Report Series 138, 1979). In genetic toxicology assays, results have generally been negative (Salmonella, *In vitro* cytogenetics, Drosophila SLRL/RT and micronucleus tests). However, positives were reported for the mouse lymphoma and *in vitro* SCE assays (although these were not particularly convincing positives).

A
B C D

As part of the ILSI program. BIPI is testing sulfisoxazole in the Tg.AC hemizygous model (responders). Both the dermal and oral (gavage) routes are being used. Briefly, groups of 15 male and 15 female Tg.AC mice (confirmed as responders by Taconic) are being treated daily for 26 weeks. For the dermal dosing, levels of 12.5, 50 and 100 mg/day are administered using 20% DMSO/80% acetone as the vehicle. These dose levels were selected based on a two week range-finder in FVB/N mice where follicular hypertrophy of the thyroid was observed at levels higher than 60 mg/day. For the gavage route, dose levels of 500, 1000, and 2000 mg/kg/day are based on a range-finder where 3000 mg/kg/day produced histopathological changes similar to those seen with the dermal range-finder. CMC/Tween 80 is the vehicle for the gavage dosing. This study started in Mid-November, 1998 and necropsy is scheduled for May, 1999.

Contact: Dr. H. Holden at (203) 798-4678
hholden@bi-pharm.com

Articles of Interest

Thompson KL, Rosenzweig BA and Sistare FD. (1998) An Evaluation of the hemizygous transgenic Tg. AC mouse for carcinogenicity testing of pharmaceuticals II. Genotyping that predicts tumorigenic responsiveness. Toxicologic Pathology 26: 548-555.

Blanchard KT, Ball DJ, Holden HE, Furst SM, Stoltz JH and Stoll RE. (1998) Dermal carcinogenicity in transgenic mice: relative responsiveness of male and female hemizygous and homozygous Tg.AC mice to 12-O-tetradecanoylphorbol 13-acetate (TPA) and benzene. Toxicologic Pathology 26: 541-547.

Characterization of Non-Responder Phenotype in Tg.AC Mice and Development of Non-Isotopic DNA RFLP Analysis in Tg.AC Mice

GJ Moser[1], DC Kantz[1], G Lacks[1], RR Tice[1], TL Goldsworthy[1], RW Tennant[2], JW Spalding[2], and R Cannon[2].

Integrated Laboratory Systems[1] and National Institute of Environmental Health[2], Research Triangle Park, NC

Tg.AC transgenic models have been proposed as a short term *in vivo* alternative or adjunct to the chronic bioassay to identify and characterize the mechanisms of action of environmental carcinogens. Tg.AC mice carry a v-Ha-ras oncogene and act as genetically-initiated mice. When topically exposed to the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA), Tg.AC mice responded with skin papillomas as a reporter phenotype.

Since 1991 in conjunction with the National Institute of Environmental Health Sciences (NIEHS), we have conducted a series of 6-month assays in Tg.AC mice with exposure to TPA as a positive control. Recently, an increase in TPA-exposed non-responder (NR) Tg.AC mice has been identified. To characterize the NR phenotype over a series of experiments, we calculated the incidence of Tg.AC mice with papillomas and mean number of papillomas per tumor-bearing mouse after exposure to TPA from 1991 to present. In these experiments, 1.25 or occasionally 2.5 µg TPA was topically administered twice weekly to group housed female mice. The incidence of papillomas in TPA-treated Tg.AC mice from 1991 to 1996 in nine experiments was consistently over 90%. In 1997, the incidence of papillomas precipitously dropped, ranging from 44% to 65%. Likewise, the mean number of papillomas per tumor-bearing mouse dropped from a minimum of over 12 papillomas in 1991-1996 to only 6.4 papillomas in 1997. These data indicate that until 1997, there was consistently a high incidence of papillomas in TPA-exposed mice. The identification of Tg.AC responder mice and a change in breeding practices is currently an active, ongoing process.

Tg.AC studies have indicated that the tumorigenic response to chemical carcinogens is dependent on the activation and sustained expression of the oncogenic v-Ha-ras transgene. However, the presence of Tg.AC mice incapable of responding with TPA-induced skin papillomas (i.e. NR) in a number of recent Tg.AC tumor studies has created the need for a sensitive and specific assay to identify NR mice (K Blanchard, D Ball, H Holden, S Furst, J Stoltz, and R Stoll, *Toxicol. Pathol*., 26:541-547, 1998) . DNA blot experiments have linked a 2000 base pair Bam H1 fragment containing two inverted copies of the zetaglobin promoter to the responder phenotype. It is believed that the deletions in the palindrome region during replication of DNA may contribute to the NR phenotype. Recently Frank Sistare (FDA) developed a ^{32}P-based Southern blot assay. This isotopic assay allows for the identification of NR based on a band shift in the 2000 base pair Bam H1 Fragment of the promoter region (K Thompson, B Rosenzweig, and F Sistare, *Toxicol. Pathol*., 26:548-55, 1998). This ^{32}P-based methodology is commonly used and is a reliable method to identify the responder and NR genotype.

To increase the efficiency of detecting NR, we have developed an analogous non-isotopic DNA Southern blot procedure. DNA from frozen tail tissue was isolated using the phenol extraction method modified by Sistare and coworkers. Approximately 10 µg of genomic DNA was digested with BamH1, precipitated, resuspended, run out on a 1.2% agarose gel, and transferred onto UV membranes. Membranes were hybridized with the Eco R1/Bam H1 zetaglobin promoter fragment of the Tg.AC transgene labeled with fluoroscein. An anti-flouroscein alkaline phosphatase conjugate was used for detection. Generally blots were placed on film for only an hour.

We have compared the responder/NR results of our non-isotopic procedure with the ^{32}P-based one and evaluated the reproducibility of the

same samples on a number of different blots. Complete concordance was found between the non-isotopic and isotopic assays and the same samples on different blots. The non-isotopic methodology has several advantages over the radioactive-based method. The non-isotopic assay requires less time than the ^{32}P-based assay primarily due to decreased development time of the blots. The time required to develop the Southern blots is reduced from 1-3 days with the isotopic procedure to 1 hour with the non-isotopic procedure. Also, the fluorescein-labeled probes have an extended half-life of 6 months relative to a half-life of 2 weeks with ^{32}P-labeled probes. The increased half-life of the probe along with ablation of the cost of radioactive waste disposal greatly decreases the cost of the non-isotopic methodology. Thus, this non-isotopic methodology is an accurate, sensitive, and reproducible genotyping procedure that requires less time and expense, and obviates the need for radioactivity. Collectively, these data indicate, that up until 1997, there were no increases in the NR phenotype, and NR can be genotypically identified by non-isotopic Southern analysis.

For technical questions on the non-isotopic Southern blot analysis for identification of the NR Tg.AC phenotype, contact Dr. Glenda Moser or Daphne Kantz at Integrated Laboratory Systems, P.O. Box 13501, Research Triangle Park, NC 27709.

Contact: Dr. G. Moser at 919-544-4589 x322 or
D. Kantz at 919-544-4589 x238

Upcoming Meetings and Events

☺ ILSI/HESI Alternatives to Carcinogenicity Committee Meeting
Washington, DC

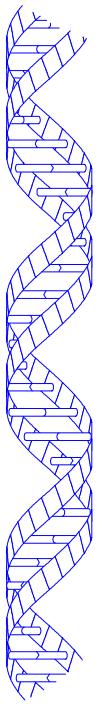
November 30 to December 1, 1998

☺ Transgenic Animals and Safety Evaluation of Pharmaceuticals and Industrial Chemicals: Scientific and Regulatory Perspectives
Baltimore, MD

December 10-11, 1998

☺ Gene Environment Interactions: Emerging Issues, Technologies and Biological Paradigms
Barton Creek Conference Resort, Austin, Texas

December 2-5, 1998



Studies on Characterization of the Tg.AC Mouse

by Don Delker, Pam Spencer, and Bhaskar Gollapudi, The Dow Chemical Co.

The usefulness of the Tg.AC mouse model as an alternative to the two year bioassay is currently being evaluated at The Dow Chemical Company using several approaches. We are interested in identifying organs, other than the skin and forestomach, that might be potential targets for tumor development following chemical exposure. We are also investigating constitutive expression of the v-Ha-ras transgene in various tissues, and the modulation of this expression in response to chemically-induced cytotoxicity.

Using an RT-PCR method and primers for the SV-40 region of the transgene, we have identified that tissues such as kidney, hind brain, and spinal cord constitutively express the transgene. Interestingly, v-Ha-*ras* expression in these tissues correlated well with GATA-3 mRNA expression, a transcription factor known to bind the zeta-globin promoter. Liver, on the other hand, did not show v-Ha-ras expression either constitutively or following chemically-induced liver injury. The lack of transgene expression in the liver correlated well with the levels of GATA-3 mRNA.

We are also investigating mechanisms involved in tumor susceptibility in the Tg.AC mouse model. We found that topical application of 2.5 mg of TPA per mouse (3 times/week, 26-weeks) resulted in paillomagenesis in approximately 50% of homo- as well as hemizygous (for the transgene) mice. After the discovery of the non-responder genotype by Dr. Sistare and colleagues, we had the mice genotyped at Taconic for the 2-kb diagnostic DNA band for tumor responsiveness and these results corresponded well with the phenotypic markers. We have also evaluated v-Ha-*ras* gene expression in responder and non-responder mice and found no difference between the genotypes in transgene expression in those tissues that constitutively express it. It is conceivable that responsiveness to v-Ha-*ras*-mediated tumorigenesis in Tg.AC mice is determined by cellular factors that are tissue specific and/or work in cooperation with the transcription of the transgene. Alternatively, it is possible that there is an inducible component among the multiple copies of the transgene that plays a key role in determining tumor responsiveness and the non-responder mice lack this component.

Results from the above studies have been submitted for publication and/or presentation at the next Society of Toxicology Annual Meeting.

Contact: Dr. B. Gollapudi at (517) 636-7179
bgollapudi@dow.com

The End

A B C D

Dept. of Toxicology and Safety Assessment
Research & Development Center
900 Ridgebury Rd./PO Box 368
Ridgebury, CT 06877-0368

The Tg.AC Workgroup Newsletter